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TITLE

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RUNNING HEAD

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ABSTRACT

Winter has long been considered a dormant season in boreal forests regarding plant physiological activity such as nutrient acquisition. However, biogeochemical data clearly show that soil can remain unfrozen with substantial rates of nutrient transformation for several weeks following autumn snowfall. Here we examined nitrate (NO_3^--N) assimilation by black spruce (*Picea mariana*) during summer and winter in Interior Alaska to test our hypothesis that this boreal species is able to assimilate NO_3^--N, even at the very low temperatures typical of early winter. Nitrate reductase activity (NRA) was measured in current year needles and fine roots of black spruce as an indicator of NO_3^--N assimilation in the summer and winter at two boreal forest sites. NO_3^--N concentration in the needles and roots were also measured to determine whether NO_3^--N was available in plant tissue for the enzyme. NRA and NO_3^--N were detected in needles and roots in the winter as well as the summer. The results of a generalized linear mixed model (GLMM) showed that season had minimal effects on NRA and NO_3^--N concentration in this species. Additionally, the effect of incubation temperature for the NRA assays was tested at 30 °C and -3 °C for samples collected in the winter. Substantial enzyme activity was detected in winter-collected samples, even in incubations conducted at -3 °C. These results indicate that this dominant tree species in the boreal forests of Interior Alaska, black spruce, has the capacity to assimilate NO_3^--N below freezing temperatures, suggesting that the physiological activity required for N resource acquisition may extend beyond the typical growing season. Our findings coupled to biogeochemical evidence for high microbial activity under the snow also indicate that winter N acquisition should be taken into account when estimating the annual N budgets of boreal forest ecosystems.

KEYWORDS

nitrate (NO_3^--N), nitrate reductase activity (NRA), non-growing seasons, Taiga

INTRODUCTION

Nitrogen is among the most important limiting factors of plant productivity in the boreal forests of Interior Alaska (Yarie and Van Cleve 2006). The annual plant N requirement is only partly supplied by the major N source for plants, soil inorganic N (Valentine et al. 2006, Lisuzzo et al. 2008). In other words, there are marked discrepancies between the current estimates of inputs of inorganic N available to plants (via N-mineralization, N-fixation, and dry/wet deposition) and their annual N uptake rates or requirements (Kielland 2001, Valentine et al. 2006, Lisuzzo et al. 2008). The direct uptake of N in the form of amino acids further narrows the growing season gap between supply and demand (Persson and Näsholm 2001, Kielland et al. 2006a). Moreover, some of the discrepancies may be explained by uptake during the shoulder seasons, i.e., the period between growing season and mid winter, which has been largely ignored in the estimation of N flux including above-mentioned works. Kielland et al. (2006b) used over-winter incubations to demonstrate that boreal forest soils have a substantial capacity for N mineralization during the cold season and concluded that conventional measures have greatly underestimated the annual flux of inorganic N because they have been restricted to the growing season (May–September). In this study, we focused on N use by boreal plants during the winter to discuss the possible contribution of winter for N acquisition by plants.

Two inorganic forms of N (nitrate [NO_3^--N] and ammonium [NH_4^+-N]) in soils are available to most plant species. NO_3^--N assimilation has been investigated in a variety of plant species using nitrate reductase activity (NRA) as an index (e.g., Smirnoff et al. 1984, Gebauer et al. 1988). Nitrate reductase (NR) catalyzes the reduction of NO_3^--N to NO_2^--N, which is the first and rate-limiting step of plant NO_3^--N assimilation. Measurements of NRA can be used to estimate plant NO_3^--N use without disturbing the soil, which is not the case for experimental manipulations, such as the application of ^{15}N tracers. Numerous studies have shown that both external environmental changes and internal physiological shifts in plants can cause temporal changes in NO_3^--N assimilation (cf. Högberg et al. 1986, Gebauer et al. 1987, Schmidt et al. 1991, Högberg et al. 1992, Stadler and Gebauer 1992, Ohlson and Högbom 1993, Pearson and Ji 1994, Troelstra et al. 1995, Koyama et al. 2008). However, these studies measured NRA during the growing seasons of the

73 species examined. NRA has rarely been examined in the winter, although Koyama et al. (2008)
74 investigated the nitrate assimilation of a temperate, evergreen *Quercus* species during the winter,
75 and the NRA of several evergreen coniferous species growing in temperate forests actually appears
76 to be higher in the winter than in the summer (M. Ueda pers. comm.).
77 In boreal forests, where winter air temperatures can fall to below -40 °C, the seasonal
78 patterns of enzyme activity may differ from warmer regions. On the other hand, Kielland et al.
79 (2006b) demonstrated that soils from black spruce stands exhibited significant nitrification in late
80 winter to spring. Hence, any potential to take up and/or assimilate NO_3^--N at very low temperatures
81 in boreal tree species will influence the current estimates of N flux in these cold, high latitude forests.
82 Furthermore, recent global changes in climate could make uptake/assimilation activity during the
83 winter of even greater relevance in nutrient budget calculations.
84 In this study, we compared winter and summer NRA and NO_3^--N concentrations in the
85 needles and fine roots of black spruce (*Picea mariana* (Mill.) Britton, Sterns and Poggenb.) in a
86 boreal forest located in Interior Alaska, USA. We also simulated ambient soil temperatures during
87 wintertime enzyme incubations to determine the extent to which enzyme activity is maintained
88 under near-natural conditions in an attempt to explore the idea that black spruce can maintain
89 physiological activities at low temperatures.
90
91 ## MATERIALS AND METHODS
92 ### *Study Site*
93 The study was conducted in late-successional black spruce forests in Interior Alaska, USA
94 (64°52′N, 147°50′W). During the study years, the temperature at the nearby weather station ranged
95 from -39.13–33.02 °C, averaging 0.37 °C (Fig. 1; Van Cleve et al. 2016). The average annual
96 precipitation at the site was 437 mm, of which 35 % fell as snow. The ground was covered with
97 snow from mid-October through late April. The mean annual NO_3 and total inorganic N deposition
98 in this site from 2009 to 2016 were 0.54 ± 0.10 and 0.99 ± 0.34 kg-N ha^{-1}, respectively, which are
99 lower by an order of magnitude than the US average (National Atmospheric Deposition Program
100 (NRSP–3) 2017; http://nadp.sws.uiuc.edu).

101

102 ***Sample Collection and Laboratory Analysis***

103 *Experiment 1: effects of season, site, and tissue on NRA and NO_3^--N concentration*

104 The first experiment to compare the effects of seasons, sites, and tissues was conducted in
105 two late-successional black spruce forests near the campus of the University of Alaska, Fairbanks,
106 and two sites were located approximately 2 km away from each other (site 1: 64°51′36″N,
107 147°53′12″W and site 2: 64°51′49″N, 147°50′43″W). Plant sample collection was conducted at site
108 1 in July (summer) and December (winter) in 2009 and at site 2 in July (summer) 2015 and
109 November (winter) 2016. At the time of summer sampling, the air temperatures in 2009 and 2015
110 were approximately 17 °C and 15 °C and the surface soil temperatures were approximately 13 °C
111 and 14 °C, respectively. During winter sampling, the air temperatures were approximately -20 °C
112 and -10 °C in 2009 and 2016, and the surface soil temperatures were about -2 °C and -1 °C,
113 respectively. The snow depth reached approximately 14 cm and 8 cm in 2009 and 2016,
114 respectively.

115 Current year needles and fine roots (diameter: < 2 mm) of black spruce were collected in
116 the summer (July 2009 and 2015) and winter (December 2009 and November 2016) from five
117 mature trees. These needles and roots were used in NRA and NO_3^--N concentration assays.

118

119 *Experiment 2: effects of incubation temperature on NRA*

120 In the second experiment to test the effects of incubation temperatures on NRAs, both
121 needle and root samples from site 1 were incubated at two temperatures: 30 °C and -3 °C. The
122 incubation at 30 °C provided optimal conditions for enzymatic catalysis. Incubation at -3 °C
123 simulated the soil temperature on the day of sampling. We did not run tests at air temperature on the
124 day of sampling (-20 °C) because this was well below the freezing temperature of the incubation
125 buffers.

126

127 *The assay of NRA($+NO_3$), NRA($-NO_3$) and NO_3^--N concentration*

128 We measured two types of NRA as indices of plant NO_3^--N use: NRA($+NO_3$) and

NRA($-NO_3$). NRA($+NO_3$) is a measure of the nitrate reduction capacity with a non-limiting nitrate supply; NRA($-NO_3$) is the nitrate reduction rate of nitrate absorbed by plants, which is considered to be the closest approximation of the *in situ* NO_3^--N assimilation rate (Thomas and Hilker, 2000). Both NRA assays were conducted with modified versions of the Jaworski procedure (Jaworski, 1971, Thomas and Hilker, 2000, Koyama and Kielland, 2011). NRA($+NO_3$) was measured as the rate of nitrite (NO_2^--N) production in an incubation buffer containing a non-limiting concentration of NO_3^--N. NRA($-NO_3$) was determined in parallel measurements using an incubation buffer without additional NO_3^--N, which allowed us to examine the relative magnitude of *in situ* NO_3^--N assimilation.

Current year needles and fine roots (diameter: < 2 mm) were sampled from five mature black spruce trees on each sampling occasion. Needle samples were collected from the surface of the crown at various heights, and the sampled needles were mostly exposed to adequate light due to low canopy density (Fujino pers. comm.). Root samples were washed in tap water and then in deionized water to remove the soil. Fine root samples were randomly collected from root tips, thus possibly ectomycorrhizal (ECM) fungal tissue were mixed with spruce root tissue. Approximately 100 mg (fresh weight) of needles and roots were cut into small fragments (each *ca*. 2 mm long) and transferred to test tubes. The incubation buffer (5 mL) was added to the needles and roots, and the tube contents were vacuum infiltrated. The composition of the incubation buffer for NRA($+NO_3$) was as follows: 0.1 mol L^{-1} KNO_3, 0.1 mol L^{-1} KH_2PO_4, 1.5 % 1-propanol; the pH was adjusted to *ca*. 7.5 using an NaOH solution. The concentration of NO_3^--N was determined by a preliminary optimization process in which different concentrations of NO_3^--N were added to the incubation buffer. A supply of varying NO_3^--N concentration ranging from 0.00 mM to 0.25 mM in incubation buffer yielded a peak of NRA at 0.10 mM of NO_3^--N supply (Appendix 1). The incubation buffer for NRA($-NO_3$) contained all of the reagents other than KNO_3. The samples were incubated for 1 h in darkness, and NO_2^--N concentration in the incubation buffer was measured at the end point. Before the measurement, enzyme activity was terminated by placing the sample vials in hot water (>80 °C). The concentration of NO_2^--N in the incubation buffer was measured colorimetrically following diazotization (Keeney and Nelson, 1982). The confounding effects of plant pigments were

accounted for by subtracting the absorbance of controls to which N-naphthylethylene diamine dihydrochloride was not added (Gebauer et al. 1998). A fraction of each leaf sample was oven-dried at 105 °C and then weighed to calculate the activity per unit dry weight.

For tissue NO_3^--N concentration measurements, the aliquots of needle and root samples were dried and ground. Approximately 100 mg of ground sample was extracted with 10 mL deionized water for 1 h at 45 °C. The extract was filtered, and the concentration of NO_3^--N in the extract was colorimetrically analyzed in an AutoAnalyzerIII (BLTec, Osaka, Japan). Plant pigments in extracts might cause an overestimation of NO_3^--N concentration, and other unknown compounds in the extracts might inhibit the reduction of NO_3^--N to NO_2^--N, which is colorimetrically measured in the AutoAnalyzerIII (data not shown). Again, the confounding effects of plant pigments were taken into account by subtracting the absorbance of controls to which N-naphthylethylene diamine dihydrochloride was not added. In addition, a standard addition method was applied to compensate for the effects of pigments and other compounds in the extract as necessary when the sample composition was unknown or complex and might affect the analytical signal (Harris 2007). In this method, standard solutions of known concentrations were added to each extract, and from the increases in signal (i.e., absorbance), the concentration in the original extract was calculated.

Statistical Analysis

For experiment 1, we fitted a generalized linear mixed model (GLMM) with a gamma distribution to evaluate the effects of Season (summer or winter), Site (site 1 or site 2), and Tissue (needle or root) on NRA($+NO_3$), NRA($-NO_3$), or NO_3 concentrations, following a Shapiro-Wilk test to test the normality of data. Five individual trees were included as random effects. Two of NRA($+NO_3$) and seven of NO_3 concentrations data were below the detection limit; to fit the model with a gamma distribution, 1×10^{-10} and 1×10^{-6} were substituted for zero for these samples that presented values below the detection limit, respectively. All possible subsets of the explanatory variables and their interactions were compared with Akaike Information Criterion (AIC) for each of the response variables, NRA($+NO_3$), NRA($-NO_3$), or NO_3^--N concentrations.

For experiment 2, we fitted a generalized linear mixed model (GLMM) with a gamma

distribution to evaluate effects of the variables Incubation temperature (-3 °C or 30 °C) and Tissue (needle or root) on NRA($+NO_3$) or NRA($-NO_3$). Five individual trees were included as random effects. All possible subsets of the explanatory variables and their interactions were compared with Akaike Information Criterion (AIC) for each of response variable, NRA($+NO_3$) or NRA($-NO_3$).

It should be noted that in both experiments, the link function 'inverse' was applied for GLMM with gamma distribution, and consequently a positive value of the coefficient implies a negative effect of the explanatory variable on the response variable. It is worth noting that all of the regression coefficients can be compared to each other, although they were not standardized, as all of the variables are categorical variables with an equal number of categories: two in each experiment. All statistical analyses were conducted using the statistical platform R (ver. 3.3.3; http://www.R-project.org), and the lme4 package (version 1.1-13) was used for fitting GLMM.

RESULTS

Experiment 1: effects of season, site, and tissue on NRA and NO_3^--N concentration

Both NRA($+NO_3$) and NRA($-NO_3$) were detected in the needles and fine roots of black spruce in experiment 1 (Fig. 2a, b). The best performing model fitted for NRA($+NO_3$) had Season, Site, and their interaction as explanatory variables. However, only Site had the coefficient with a P-value lower than 0.05, indicating that zero was not included within the 95 % Wald confidence interval (CI) of estimated coefficient (Table 1). The best performing model fitted for NRA($-NO_3$) had all of the explanatory variables and their interactions except the interaction of Season × Site × Tissue. However, the coefficient for the Season and the interaction Season × Tissue exhibited P-values higher than 0.05, indicating that zero was included within the 95 % Wald CI of estimated coefficients.

NO_3^--N was also detected in most needle and fine root samples (Fig. 2c). The best performing model fitted for NO_3^--N concentration had Season, Tissue, and their interaction as explanatory variables. However, Season and Tissue had a P-value higher than 0.05, indicating that zero was included within the 95 % Wald CI of estimated coefficients.

213 ***Experiment 2: effects of incubation temperature on NRA***

214 Both NRA(+NO_3) and NRA(-NO_3) were detected in current year needles and fine roots,
215 even at the low incubation temperature (-3 °C; Fig. 3). Both the Incubation temperature and Tissue
216 were selected for the best performing model fitted for NRA(+NO_3), but their interaction was not
217 (Table 2). Moreover, both the coefficient for the Incubation temperature and Tissue showed P-values
218 lower than 0.05, indicating that zero was not included within the 95 % Wald CI of estimated
219 coefficient. On the other hand, the best performing model for NRA(−NO_3) had only Incubation
220 temperature as a coefficient, and the P-value for that was higher than 0.05, indicating that zero was
221 included within the 95 % Wald CI of estimated coefficient.

222

223 DISCUSSION

224 ***Nitrate Assimilation of Black Spruce in Winter and Summer***

225 Winter is generally considered to be a season of dormancy in boreal forests due to the
226 extremely low temperatures, reduced light intensity, and short photoperiods. However, we have
227 demonstrated that black spruce trees in Interior Alaska are able to assimilate NO_3^--N in the winter as
228 well as in the summer (Fig. 2), indicating that, in the winter, (i) black spruce induced NR and (ii)
229 NO_3^--N was available for NR.

230 The disparity between our findings and the accepted winter dormancy concept could be
231 attributed to a *de novo* induction of the enzyme during the storage time in our experiments. However,
232 the *in vivo* NRA was measured under the premise that NR had not been induced *de novo* during the
233 storage period after sample collection. This premise was based on the known light requirement for
234 NR induction (Lillo et al. 2004); our samples were stored in complete darkness. Furthermore,
235 Högberg et al. (1986) found that the shoot NRA of *Deschampsia flexuosa* declined for the first 30
236 min of storage and remained stable thereafter. Thus, we suggest that NR was not newly induced in
237 our detached samples during storage, and that the NRA detected by our measurements was not likely
238 to be the result of artificially inflated rates of enzyme induction following sample collection.

239 The results of GLMM fitting and model selection showed that Season had no significant
240 effect on NRA(+NO_3), NRA(−NO_3), or NO_3^--N concentration (Table 1). Site and the interaction

241 between Season × Site were selected as effective variables in the best models for both of
242 NRA(+NO_3) and NRA(−NO_3) (Table 1). In addition, Tissue and the interaction between Site ×
243 Tissue were also selected in the best model for NRA(−NO_3). On the other hand, only the interaction
244 between Season × Tissue was selected as a variable influencing NO_3^--N concentration. Taken
245 together, these results indicated that season was not a significant factor affecting NO_3^--N use by
246 black spruce. Because newly expanding leaves contain higher concentrations of N than fully
247 expanded leaves, winter buds of temperate evergreen species most likely receive N transported from
248 old tissues (Silla and Escudero 2003, Koyama et al. 2008). Consequently, we surmise that the
249 wintertime acquisition of N by the needles of black spruce may play a role in the preparation of
250 additional N sources for the new needles that flush in late spring in these sites.
251 Lambers et al. (2008) demonstrated that the NRA shoot/root ratio generally increases with
252 NO_3^--N availability in temperate and subtropical species. Our results showed that black spruce
253 assimilate nitrate in their current year needles. Some previous studies revealed prior assimilation of
254 nitrate in the roots of coniferous species (Peuke and Tischner 1991, Gebauer and Schulze 1997, Yao
255 et al. 2011), and our results were contrary to these observations. Assuming soil NO_3^--N availability
256 was lower at site 2 based on the site differences of NRAs, the results were consistent with the
257 relationship between the allocation of NRA and soil NO_3^--N availability in temperate and
258 subtropical species (Lambers et al. 2008), because both NRA(+NO_3) and NRA(−NO_3) were higher
259 in roots than in needles at site 2.
260
261 ***Effects of Incubation Temperature on Winter NRA***
262 NRA(+NO_3) was detected even at the low incubation temperature, -3 °C, both in needles
263 and roots (Fig. 3), although the low incubation temperature significantly reduced the activity in
264 comparison with the samples incubated at the high temperature (30 °C; Table 2). Roots showed
265 lower NRA(+NO_3) than needles, and this allocation pattern did not differ by the incubation
266 temperature. On the other hand, neither incubation temperature nor tissue influenced NRA(−NO_3),
267 implying the low incubation temperature did not inhibit NRA(−NO_3). Thus, the enzyme in winter
268 needles is clearly capable of catalyzing NO_3^--N reduction at very low temperatures.

Early studies attempted to optimize the incubation conditions for *in vivo* NRA assays (Nicholas et al. 1976, Al Ghabi and Hipkin 1984, Gebauer et al. 1984). Optimal temperatures were found to be in the range 28–33 °C (Sym, 1984, Högberg et al. 1992), and even higher optimal temperatures (40–50 °C) were reported for some crop species (Chopra, 1983). Högberg et al. (1992) showed that NRA increased with temperature, reaching an optimum at approximately 25 °C. At the coldest incubation temperature they applied (approximately 0 °C), the NRA was very low (Högberg et al. 1992). However, these results were obtained from temperate species and/or herbaceous taxa, such as barley (*Hordeum vulgare*) and *D. flexuosa*. No trials were conducted on boreal evergreen tree species. Furthermore, earlier experiments were conducted during the growing season, but never in winter. The distinct NR responses to temperature in boreal species may well represent an adaptation to cold climates.

We tested only two temperatures in our study, which did not allow us to examine the functional responses of the NR enzyme to temperature. We were also unable to measure enzyme activity in the needle samples at ambient winter air temperatures (-20 °C), which would have frozen the incubation buffer. Accordingly, we cannot rule out the possibility that we overestimated the activity of the enzyme in winter needle samples. Nevertheless, our study has reduced the level of NRA overestimation attributable to the conventional incubation temperature (30 °C).

Ecological Implications

Based on the results showing the capacity of black spruce to use NO_3^--N, we conclude that this species is able to assimilate NO_3^--N in the winter. In earlier studies that showed significant species difference in the capacity to assimilate nitrate, coniferous species or gymnosperms were considered to have low capacities for nitrate assimilation (Smirnoff et al. 1984, Gebauer et al. 1998, Hayashi-Tang et al. 2012), and the our results were consistent with these earlier studies. However, considering that black spruce maintained the capacity to assimilate nitrate in winter, this observation indicates that they are capable of using nitrate for a longer period than deciduous species.

We were not able to estimate the magnitude of NO_3^--N uptake in winter from our results of NRA and NO_3^--N concentration, because NO_3^--N, unlike NH_4^+-N can be stored in plant tissues. It is

297 possible that the plants had absorbed and stored NO_3^--N during previous seasons and then
298 subsequently assimilated the stored ions during the following winters. Our results showed lower root
299 NO_3^--N concentration in the winter than in the summer (Fig. 2c), and this may be a consequence of
300 plant usage of internally stored NO_3 in winter. The quantitative evaluation of winter N acquisition to
301 the whole N budget requires further investigation.

302 Kielland et al. (2006b) suggested that the restriction of soil process measurements to the
303 growing season greatly underestimated the annual flux of soil N in Interior Alaska. Our data
304 corroborate this viewpoint; the N assimilation that we measured during the winter strongly indicated
305 that the annual N budgets of boreal ecosystems should be reexamined.

306 The assimilation of NO_3^--N is an energy consuming process (Bloom et al. 1992). When
307 excess light energy is available beyond that required for carbon assimilation, it may be used for
308 NO_3^--N assimilation, thereby reducing the damaging effects of photoinhibition. The putative winter
309 NO_3^--N assimilation of boreal black spruce may function as a sink for the surplus light energy
310 absorbed by photosynthetic pigments. This proposal is certainly worthy of further exploration,
311 especially considering climate change may alter the relationship between temperature and light
312 condition.

313 New information on plant physiological performance during winter, such as photosynthesis
314 (Miyazawa and Kikuzawa 2004, Saarinen et al. 2016) and N use (Koyama et al. 2008, Onipchenko
315 et al. 2009, Ueda et al. 2010) is relevant to current considerations of recent climate change (Makoto
316 et al. 2014, Sanders-Demott and Templer 2017). The effects have generally been considered in terms
317 of the direct influence of higher temperatures, changes in habitat availability, and extensions of plant
318 growth periods (Walther et al. 2002, Cleland et al. 2007, Bokhorst et al. 2008, Miller-Rushing and
319 Primack 2008, Polgar and Primack 2011). With the current lack of information on the responses of
320 plant NRA to the range of temperatures during the boreal winter, we are not in a position to estimate
321 the influence of shorter and warmer winters on plant N acquisition. However, our data clearly show
322 that the influence of a changing winter climate on ecosystem N budgets should be taken into account
323 in considerations of the effects of climate warming.
324

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FIGURE CAPTIONS

Figure 1: Temperature changes in 2009, 2015, and 2016. The temperatures recorded at an adjacent long-term ecological research (LTER) site (64°44′30″N, 148°18′50″W) are presented as daily means (closed circles: 2009, open circles: 2015, open squares: 2016) and ranges (continuous bars: 2009, dashed bars: 2015, dotted bars: 2016). Arrows indicate the sampling days (closed arrows: 2009, open arrows: summer 2015 and winter 2016). Data were obtained from the Bonanza Creek LTER Database.

Figure 2: Seasonal differences in nitrate reductase activities (NRA) and NO_3^--N concentration in current year needles and fine roots of black spruce (*P. mariana*) in two sites. (a) NRA(+NO_3) assayed with incubation buffer containing NO_3^--N, (b) NRA(−NO_3) assayed with incubation buffer containing no NO_3^--N, and (c) NO_3^--N concentration. Both NRA measurements were made at 30 °C. Samples were collected from five individual trees in each site.

Figure 3: The effects of incubation temperature on NRA in current year needles and fine roots of black spruce (*P. mariana*) collected in December 2009 and (a) supplied with NO_3^--N [NRA(+NO_3)], or (b) not supplied with NO_3^--N [NRA(−NO_3)]. Samples were collected from five individual trees in site 1.

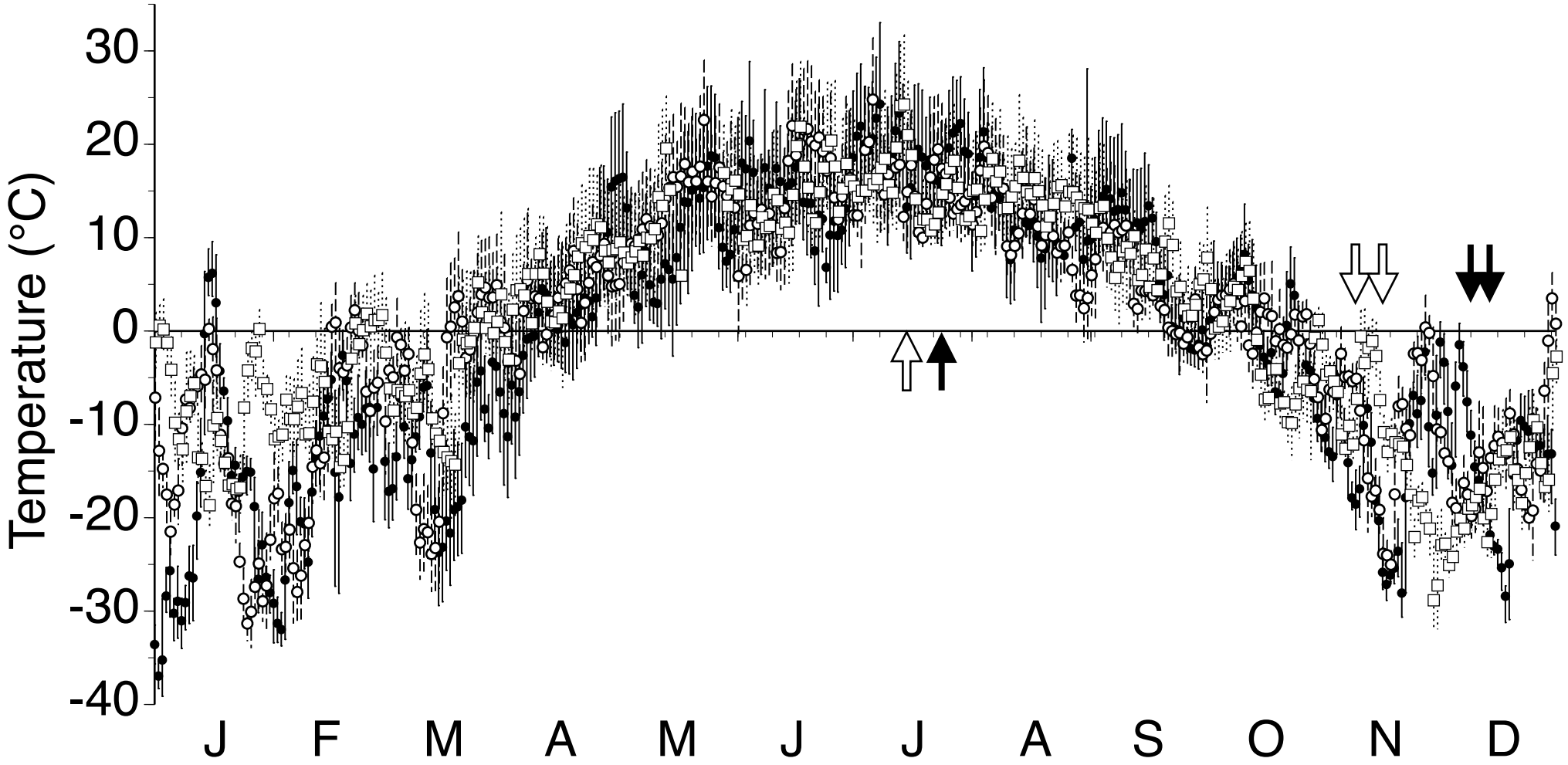


Fig. 1

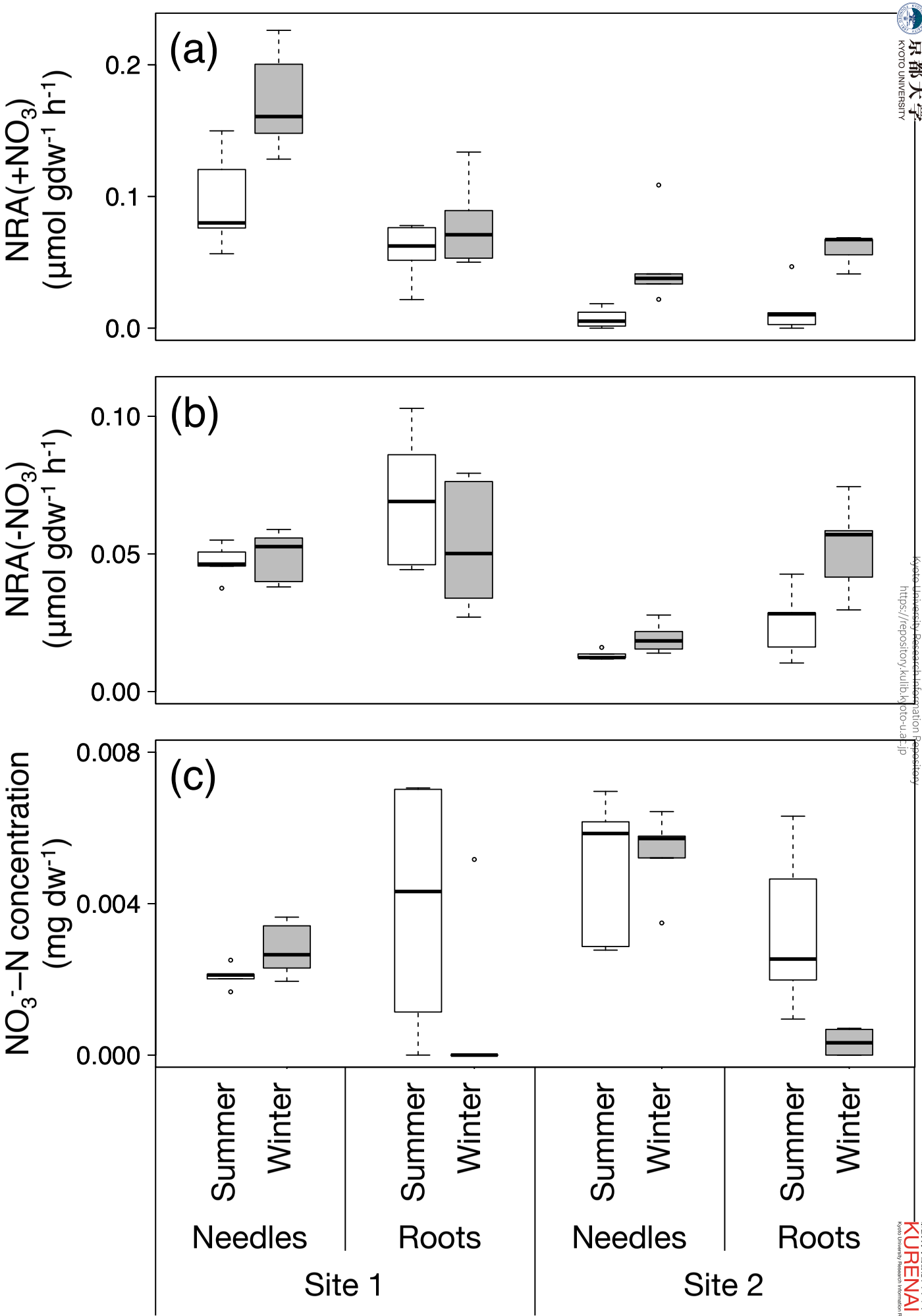


Fig. 2

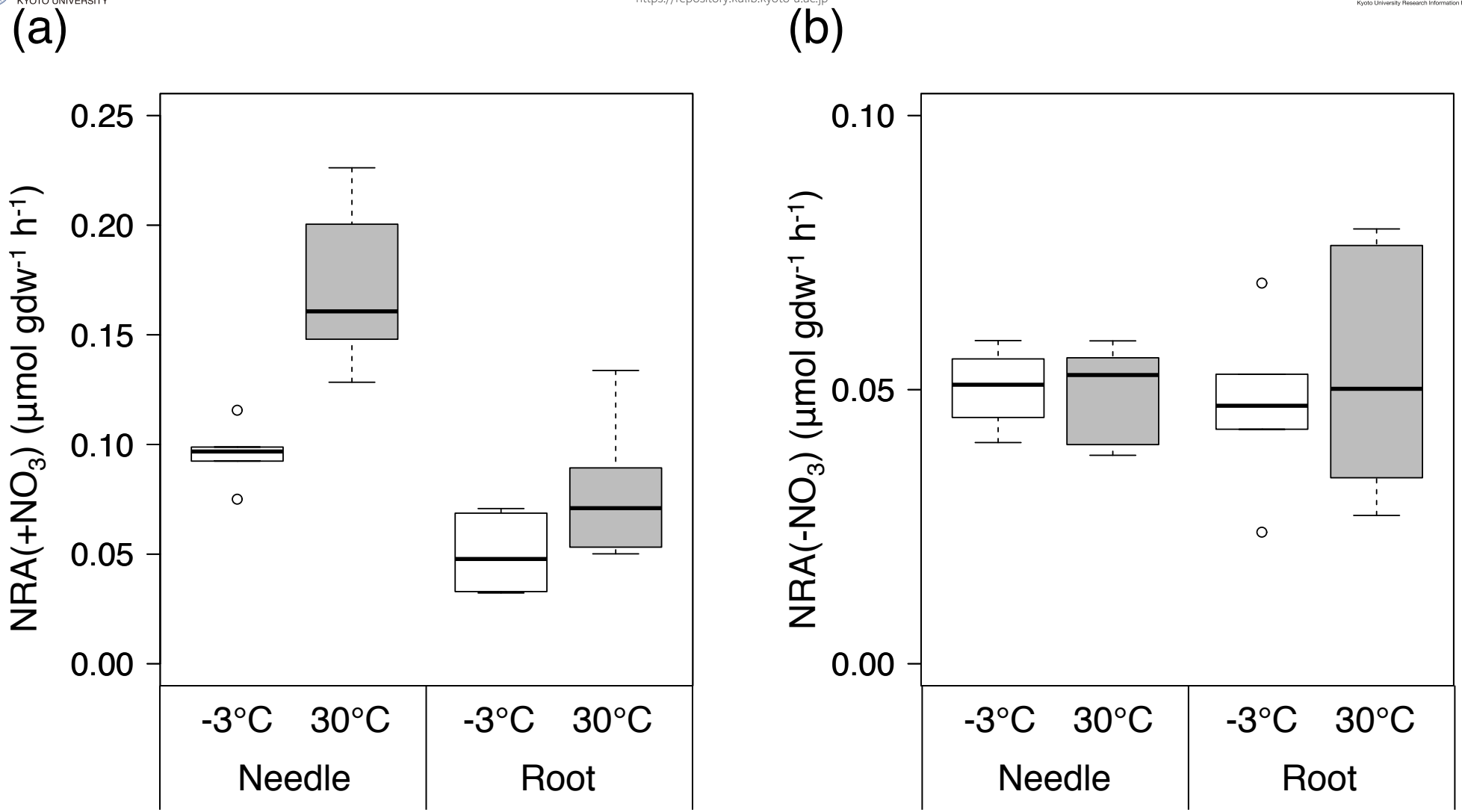


Fig. 3

Table 1 Explanatory variables and the coefficients of variables for the generalized linear mixed model (GLMM) to describe the effects of season, site, and tissue on NRA(+NO3), NRA(-NO3) and NO_3^--N concentration in black spruce. The coefficients for the best performing models are shown, and the models were selected to have the lowest Akaike Information Criterion (AIC) by comparing AIC for each of possible subset of explanatory variables (See Appendices 2–4 for details). P values indicate the probability of including zero value of coefficients within 95 % Wald confidence interval.

	Variable type	NRA(+NO_3)			NRA(-NO_3)			NO_3^--N concentration		
		Coefficient	Std Error	P value	Coefficient	Std Error	P value	Coefficient	Std Error	P value
(Intercept)		12.94	5.24	0.014	23.17	4.51	< 0.001	285.20	93.70	0.002
Season†	categorical	-5.01	6.15	0.415	-0.29	6.09	0.962	-38.84	127.45	0.761
Site††	categorical	78.78	37.17	0.034	53.52	7.14	< 0.001		-	
Tissue†††	categorical		-††††		-6.55	2.35	0.005	-7.16	134.99	0.958
Season × Site	interaction	-68.30	38.04	0.073	-23.85	8.20	0.004		-	
Site × Tissue	interaction		-		-29.38	4.82	< 0.001		-	
Season × Tissue	interaction		-		4.95	3.33	0.137	1139.10	572.22	0.047
Season × Site × Tissue	interaction		-			-			-	

†: The regression parameter estimates for these categorical variables were measured as departures from summer to winter.

††: The regression parameter estimates for these categorical variables were measured as departures from site 1 to site 2.

†††: The regression parameter estimates for these categorical variables were measured as departures from current year needles to fine roots.

††††: Variables with blank (-) in coefficients were not used in the selected best performing model based on AIC.

Table 2 Explanatory variables and the coefficients of variables for the generalized linear mixed model (GLMM) to describe the effects of incubation temperature and tissue on NRA($+NO_3$) and NRA($-NO_3$) in black spruce. The coefficients for the best performing models are shown, and the models were selected to have the lowest Akaike Information Criterion (AIC) by comparing AIC for each of possible subset of explanatory variables (See Appendices 5–6 for details). P values indicate the probability of including zero value of coefficients within 95 % Wald confidence interval.

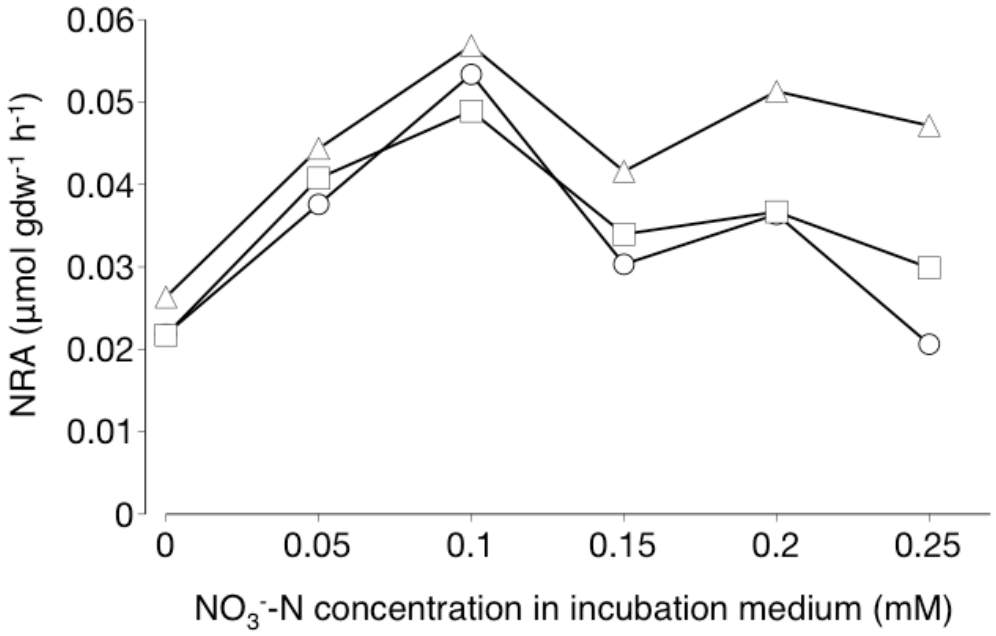
	Variable type	NRA($+NO_3$)			NRA($-NO_3$)		
		Coefficient	Std Error	P value	Coefficient	Std Error	P value
(Intercept)		10.98	1.28	< 0.001	21.80	2.50	< 0.001
Incubation temperature†	categorical	-5.04	1.53	0.001	-0.26	1.76	0.883
Tissue††	categorical	7.45	1.36	< 0.001		-	
Incubation temperature ×Tissue	interaction		-†††			-	

†: The regression parameter estimates for these categorical variables were measured as departures from the incubation temperature -3 °C and 30 °C.

††: The regression parameter estimates for these categorical variables were measured as departures from current year needles to fine roots.

†††: Variables with blank (-) in coefficients were not used in the selected best performing model based on AIC.

Appendix 1: Effects of NO_3^--N concentration in incubation buffer on needle NRA of black spruce (*P. mariana*). The incubation buffers had same composition other than NO_3^--N concentration. Different symbols indicate different individual trees (n = 3).



Appendix 2 Comparison of candidate generalized linear mixed models (GLMM) to test the effects of season, site, tissue, and their interactions on NRA(+NO_3) of black spruce. The variables (fixed effects) and their interactions applied in each model are indicated by "+", and the models are rank ordered from most to least supported based on AIC.

Season	Site	Tissue	Season × Site	Site × Tissue	Tissue × Season	Season × Site × Tissue	df	AIC	ΔAIC
+	+		+				34	-155.33	
+	+	+	+				33	-154.15	1.18
+	+	+	+	+			32	-152.80	1.35
+	+						35	-152.17	0.63
+	+	+	+		+		32	-152.15	0.02
	+						36	-151.94	0.20
+	+	+					34	-150.89	1.06
+	+	+	+	+	+		31	-150.81	0.08
	+	+					35	-150.74	0.07
+	+	+		+			33	-149.48	1.26
	+	+		+			34	-149.37	0.11
+	+	+	+	+	+	+	30	-149.33	0.04
+	+	+			+		33	-148.94	0.38
+							36	-148.14	0.80
+	+	+		+	+		32	-147.59	0.55
+		+					35	-146.99	0.61
		+					36	-146.55	0.44
+		+			+		34	-145.02	1.52

Appendix 3 Comparison of candidate generalized linear mixed models (GLMM) to test the effects of season, site, tissue, and their interactions on NRA(-NO_3) of black spruce. The variables (fixed effects) and their interactions applied in each model are indicated by "+", and the models are rank ordered from most to least supported based on AIC.

Season	Site	Tissue	Season × Site	Site × Tissue	Tissue × Season	Season × Site × Tissue	df	AIC	ΔAIC
+	+	+	+	+	+		31	-239.95	
+	+	+	+	+			32	-239.82	0.13
+	+	+	+	+	+	+	30	-237.96	1.86
+	+	+		+	+		32	-236.32	1.65
+	+	+		+			33	-236.31	0.01
	+	+		+			34	-235.32	0.99
+	+	+	+				33	-217.90	17.42
+	+	+	+		+		32	-215.90	2.00
+	+	+					34	-212.09	3.81
	+	+					35	-211.70	0.39
+	+	+			+		33	-210.10	1.59
+	+		+				34	-208.12	1.98
		+					36	-206.09	2.03
+		+					35	-205.50	0.59
+		+			+		34	-203.51	1.99
	+						36	-202.83	0.69
+	+						35	-202.27	0.56
+							36	-195.16	7.11

Appendix 4 Comparison of candidate generalized linear mixed models (GLMM) to test the effects of season, site, tissue, and their interactions on NO_3^--N concentration of black spruce. The variables (fixed effects) and their interactions applied in each model are indicated by "+", and the models are rank ordered from the most to least supported based on AIC.

Season	Site	Tissue	Season × Site	Site × Tissue	Tissue × Season	Season × Site × Tissue	df	AIC	ΔAIC
+		+			+		33	-388.70	
		+					35	-387.25	1.45
+	+	+			+		32	-387.24	0.01
	+						35	-386.70	0.54
+							35	-386.58	0.12
+	+	+		+	+		31	-386.28	0.31
	+	+					34	-385.88	0.39
+		+					34	-385.86	0.03
+	+	+	+		+		31	-385.25	0.61
+	+						34	-385.24	0.01
	+	+		+			33	-384.67	0.57
+	+	+	+	+	+		30	-384.58	0.09
+	+	+					33	-384.46	0.12
+	+	+	+	+	+	+	29	-383.60	0.86
+	+		+				33	-383.35	0.25
+	+	+		+			32	-383.25	0.10
+	+	+	+				32	-382.56	0.70
+	+	+	+	+			31	-381.37	1.18

Appendix 5 Summary of generalized linear mixed models (GLMM) comparisons to test the effects of incubation temperature, tissue, and their interactions on NRA(+NO_3) of black spruce. Models are rank ordered from the most to least supported based on AIC.

Incubation temperature	Tissue	Incubation temperature × Tissue	df	AIC	ΔAIC
+	+		15	-80.40	
+	+	+	14	-79.21	0.23
	+		16	-76.17	0.05
+			16	-62.37	1.45

Appendix 6 Summary of generalized linear mixed models (GLMM) comparisons to test the effects of incubation temperature, tissue, and their interactions on NRA(-NO_3) of black spruce. Models are rank ordered from the most to least supported based on AIC.

Incubation temperature	Tissue	Incubation temperature × Tissue	df	AIC	ΔAIC
	+		16	-109.62	
+			16	-109.61	0.01
+	+		15	-107.63	1.98
+	+	+	14	-106.25	1.38